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AWARD NUMBER: W81XWH-08-1-0280

TITLE: Metabolic Reorganization in Breast Cancer Epithelial Cells: Role of the Pentose Phosphate Shunt

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REPORT DATE: May 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 May 2009	Annual	1 May 2008 – 30 Apr 2009

4. TITLE AND SUBTITLE

Metabolic Reorganization in Breast Cancer Epithelial Cells: Role of the Pentose Phosphate Shunt

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0280

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Florida
Gainesville, FL 32611

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Breast cancer is the most frequently diagnosed cancer in women with about 180,000 new cases reported each year. Early detection allows the greatest chance for successful therapies which include surgical procedures, irradiation, hormonal, and chemical intervention. Yet, these do not always achieve complete recovery, so our goal was to develop novel techniques that might identify markers that would allow us to construct metabolic maps in different types of breast cancers to predict efficacy of therapeutic treatment options. Using NMR, we have now demonstrated that hypoxic treatment of a basal B, triple negative breast cancer cell line increases substantially the flux of non-glycolytic products into the TCA cycle which might increase the ability of cells to use oxidative phosphorylation for ATP production. If we can identify these products, we may be able to block their entry into TCA cycle to compromise the cells ability to supplement their energy and block their selective advantage.

15. SUBJECT TERMS
Metabolomics; Breast Cancer; Pentose Phosphate Shunt, Glucose Metabolism

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction:

Breast cancer is the most frequently diagnosed cancer in women in the United States, with over 180,000 new cases predicted for 2008 (American Cancer Society, Breast Cancer Facts and Figures). Breast cancer is the second leading cause of cancer death and during 2008, it is predicted that 40,480 women will die from the disease. This figure equates to about one death every 13 minutes. Early cancer detection allows the greatest chance for successful therapy which includes surgical procedures, irradiation, hormonal, and chemical intervention. Despite recent advances in diagnosis and therapeutics, complete recovery is not always realized. Therefore, novel approaches towards predicting therapeutic success and/or outcome of breast cancer patients are sought. Most of these approaches required tissue derived from tumors to detect the expression of the "family" of genes or specific proteins [1]. In our proposal, we plan an approach using nuclear magnetic resonance spectroscopy (NMR) to evaluate metabolic intermediates in human breast cancer cells (hBrC) by isotopomer analysis. The plan was to be able to construct metabolic maps (tumorigenic metabolomes) to identify unique metabolic programs that are necessary for the developing metastatic phenotype of hBrC.

Body:

Statement of Work: *Task 1*

Task 1 described our efforts to gain approval for animal usage which is planned for year three. We were successful in this, and have received the renewal for the second year of the grant. We still do not plan on using animals until the final year, and thus have no progress to report on this (see below).

Statement of Work: *Task 2*

Task 2 described our approach to compare the contribution of glycolysis, the pentose phosphate shunt (PPS), and oxidative metabolism to glucose metabolism in hBrC using ^{13}C-labeled glucose (months 1 - 15). Because of our familiarity with the MDA-MB-231 breast cancer line, we utilized these cells to establish an appropriate set of conditions which can be used across several cell types. This turned out to be more problematic than expected. The preliminary data, which compared the isotopomer pattern of glutamate in MCF10A cells (the controls) with T47D (a luminal, ER positive breast cancer cell line) and MDA-MB-231 cells (a basal B, triple negative breast cancer cell line) was conducted using a four hour pulse of [U-^{13}C]glucose which revealed that the anapleurotic reactions of the TCA cycle were down-regulated in the breast cancer lines, relative to the control epithelial line. These were also cells that were allowed to grow past confluence to provide the greatest signal to noise ratio for the NMR analysis. While Task 2 goals included using conditions that allowed the evaluation of the effect of inhibition of the PPS and activation of the pyruvate dehydrogenase complex (PDC), Task 3 required testing the effect of hypoxia. Thus, the experimental conditions for Task 2 had to match those for Task 3. Hypoxia induction requires both transcriptional and translational activation, which takes time. In previous experiments, we have demonstrated that 16h of reduced oxygen is sufficient to alter metabolic activity and protein expression [2]. Further, subconfluent cells showed the highest level of changes in protein induction. To introduce glucose (either fresh ^{12}C-glucose or ^{13}C-glucose), we would have to interrupt the hypoxic time frame at some point. Our first attempt was to add fresh glucose at the 16h time point after a complete schedule of hypoxia, and then re-introducing hypoxia for an additional four hours, similar to the preliminary data. Before going to the expense of NMR, we just examined the effect of hypoxia on lactic acid secretion into the medium.

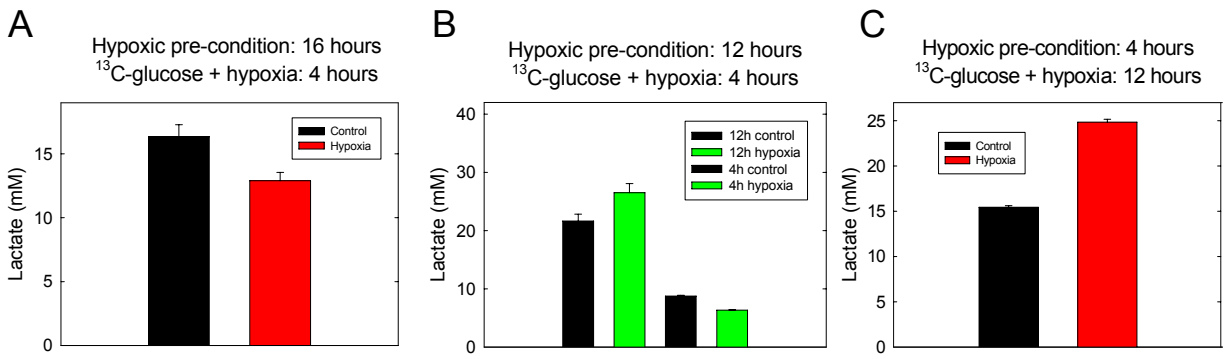

Figure 1 Effect of varying time of hypoxia and fresh glucose on lactate production: ***Panel A***: Subconfluent MDA-MB-231 cells were bathed in DMEM containing 10% FBS while being exposed to 16h of hypoxia (1% oxygen). The cells were then washed with PBS and fresh glucose (15mM) was added for an additional 4 hours with re-exposure to reduced oxygen. Data represent a single experiment run in triplicate ± SD. ***Panel B***: Subconfluent MDA-MB-231 cells were bathed in DMEM containing 10%FBS while being exposed to hypoxic conditions for 12h. This was followed by washing and re-exposing to fresh glucose and reduced oxygen for 4 hours. These data represent a single experiment run in triplicate ± SD. ***Panel C***: Subconfluent MDA-MB-231 cells were bathed in DMEM containing 10% FBS while being exposed to 4h of hypoxia (1% oxygen). The cells were then washed with PBS and fresh glucose added for an additional 12 hours with re-exposure to reduced oxygen. These data represent two independent experiments run in triplicate ± SD.

In the experiments in Figure 1, we were looking to identify conditions that would allow hypoxic exposure while maintaining elevated lactic acid production, a key measure for enhanced glycolytic activity. It was to our surprise, that in two of the conditions that we tried, that lactic acid production was actually lower in hypoxic cells (Panels A and B). However, we were able to define conditions that did allow increased lactic acid secretion, while providing an opportunity to provide isotopically labeled glucose for subsequent NMR analysis (Panel C). We extended these data by confirming that glucose utilization was increased (i.e., glucose was lost from the medium) concurrently with the increase in lactic acid (Figure 2).

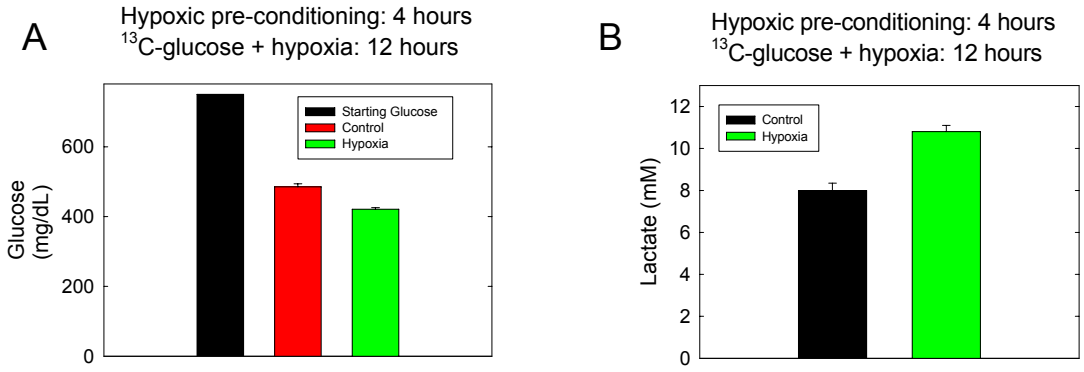

Figure 2. Glucose metabolism increased in response to hypoxia in MDA-MB-231 cells concurrently with lactic acid production. Subconflulent MDA-MB-231 cells were exposed to reduced oxygen or not for 4 hours, washed with phosphate buffered saline (PBS), and subsequently exposed to reduced oxygen or not for an additional 12 hours in the presence of 15mM glucose. Glucose remaining in the medium after 12 hours of hypoxia (or not) was compared to the starting concentration (Panel A). Lactic acid concentration, which was below the level of detection in the original medium, was measured after the final 12 hours (Panel B). These data represent a single experiment run in triplicate ± S.D.

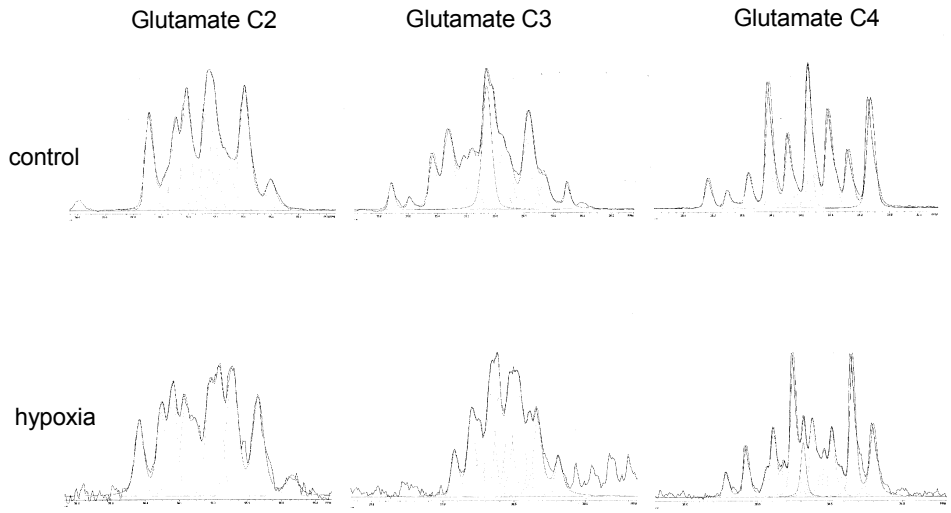

Figure 3 Isotopomer patterns of glutamate resonances in MDA-MB-231 cells in response to hypoxia: Cells from Figure 2 were washed in ice-cold saline to remove extracellular medium, extracted with ice-cold methanol, and scraped into separatory flasks. The methanolic cell slurry was diluted with an equal volume of chloroform and vigorously shaken. Ice-cold water was added and mixed to induce phase separation. The aqueous portion was collected and treated with Chelex-100, lyophilized, and resuspended in 0.5mL of D_2O. Proton-decoupled ^{13}C spectra was acquired at 500mHz in a Bruker instrument.

With the cells treated at in Figure 2, we were able to collect high-quality spectra from their extracts. Shown in Figure 3 are the glutamate carbon isotopomer patterns using direct detection. There were two noticeable differences that stood out. First, the signal to noise ratio was better in the control cells than the hypoxic cells. This indicated that the concentration of glutamate was significantly lower in hypoxic cells versus controls. Secondly, the isotopomer patterns were quite different between the two conditions. Nevertheless, we were able to model the glutamate data using tcaCALC (University of Texas, Southwestern) (Figure 4). The model that best fits the

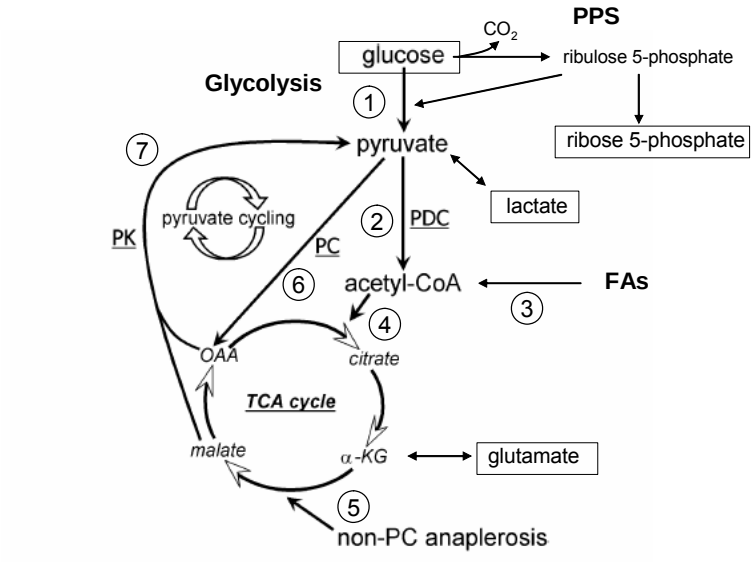

Table 1 Relative Flux Rates MDA cells

	Cont	Hyp
1	0.84	0.63
2	0.84	0.95
3	0.16	0.06
4	1.00	1.00
5**	0	0.43
6	0.69	0.37
7	0.69	0.69

Figure 4 Isotopomer Modeling using tcaCALC

control (normoxic) data uses a single pyruvate pool with a single anapleurotic entrance (pyruvate carboxylase). The model that best fits the hypoxic data uses a single pyruvate pool with two anapleurotic entrances...one via pyruvate carboxylase and one via an unidentified source (Step 5** in Table 1, above). In Figure 4 we show a diagram depicting the steps modeled in the tcaCALC modeling program. For each step that is numbered, the flux data for each of these steps are shown in Table 1. Step 1 reflects the contribution to the pyruvate pool from ^{13}C-labeled glucose. These carbons can derive from glucose via glycolysis or the pentose phosphate shunt. Step 2 relects the contribution of the pyruvate pool to the acetyl CoA pool via pyruvate dehydrogenase (in the illustration this is labeled as PDC (Pyruvate Dehydrogenase Complex). Step 3 reflects the contribution of fatty acid oxidation to the acetyl CoA pool (this pool will be unlabeled under our conditions). Step 4 reflects the contribution of acetyl CoA to the TCA cycle. By convention, this rate is defined at 1. All data are reported relative to this step. Step 5 reflects the contribution to the TCA cycle from intermediates other than pyruvate. This pool could reflect a number of amino acids, but glutamine has become a prime candidate in cancer cells. Step 6 reflects the contribution to the TCA cycle from pyruvate via pyruvate carboxylase. Step 7 reflects pyruvate recycling via PEP carboxykinase and pyruvate kinase. While these data represent only a single experiment, the do demonstrate that there are significant metabolic consequences to hypoxia. Hypoxic cells depress the amount of pyruvate that comes into the TCA pool, but an alternative substrate (presently unidentified) which in part compensates and maintains to some degree the activity of the TCA cycle which would provide some of the cellular ATP through oxidative phosphorylation. We hope to discover the identify of this unknown pool as it may be a marker for hypoxia.

To continue the work of Task 2, we will turn to the SUM line cells that were initially proposed for use in this study and the effect that inhibition of the pentose phosphate shunt has on this process, as well as enhancing the activity of PDC on the isotopomer patterns described above. We will be looking closely at the contribution by the unknown anapleurotic pool.

Statement of work: *Task 3 (months 12-21)*

Task 3 was to determine the effect of hypoxia on isotopomer patterns. We have accomplished this in part by looking at the effect of hypoxia on the MDA-MB-231 cells (see above), but will focus now on the SUM lines.

Statement of work: *Task 4 (months 21-36)*

Task 4 was planned for the third and final year of the grant where we would study metabolic flux in xenograph tumors generated from breast cancer cells. These studies are yet to be accomplished.

Key Research Accomplishments:

During this first year, we recruited a talented undergraduate student (Sue-Wei Luu) to work with us and her work is shown in Figures 1 and 2 of the body of the progress report. Sue-Wei won an ACS Summer Research Grant for this work giving her an opportunity to continue her studies with the SUM cancer cells lines. Through her work, we have defined the appropriate conditions for our cell culture experiments which should now allow us to forge ahead, unimpeded with the SUM lines.

Reportable Outcomes:

We have no manuscripts at the present time to report. However, we expect that we will be able to proceed rapidly now toward that goal.

Conclusions:

We have made good progress to our goals having completed Task 1 and initiated the experiments defined in Tasks 2 and 3. We have set up a uniform system that can be utilized for analysis of ^{13}C-isotopomers across multiple cell types. We have shown that hypoxia treatment of a triple negative cell type decreases flux from glycolysis through the TCA but ramps up an anapleurotic step to engage the down-regulated TCA cycle, likely to increase ATP synthesis via oxidative phosphorylation. We are poised now to examine the role of the pentose phosphate shunt and the activation of the pyruvate dehydrogenase complex on the metabolic pattern in breast cancer cells.

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[2] Li,Y., Wang,H., Oosterwijk,E., Tu,C., Shiverick,K.T., Silverman,D.N., & Frost,S.C. (2009) Expression and Activity of Carbonic Anhydrase IX Is Associated with Metabolic Dysfunction in MDA-MB-231 Breast Cancer Cells. *Cancer Investigation* **10.1080/06357900802653464**.